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Lactoferrin and Activated Protein C: Potential Role in Prevention of Cancer Progression and Recurrence

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ABSTRACT

Existing therapeutic interventions for controlling cancer are limited and associated with side effects. Furthermore, the recurrence of cancer poses a significant challenge to the cure of cancer. Therefore, avenues are wanted to find novel therapies for cancer treatment and cancer recurrence. In this review, we have highlighted that lactoferrin (LF) and activated protein C (APC) carry enormous potential in cancer treatment. Studies have shown that the decreased level of APC and impaired function of APC are associated with cancer progression and cancer-related mortality. Moreover, APC plays an important role in preventing prothrombotic state-mediated cancer progression and deaths. LF can also inhibit the progression of cancer by controlling the generation of reactive oxygen species, triggering the apoptosis of cancer cells, arresting the cell cycle and hindering the angiogenesis process. Additionally, APC and LF could have the potential to inhibit neutrophil extracellular traps (NETs) formations which are involved in cancer progression and the reawakening of dormant cancer cells. Hence, in this review, the anticancer potential and mechanism of APC and LF along with their potential to mitigate inflammation and NETs-mediated cancer progression and recurrence has been discussed. Additionally, possible future strategies to develop effective and safe anticancer treatment using LF and APC have also been discussed in this review.

Keywords: Cancer, activated protein C, Lactoferrin, neutrophil extracellular traps, cancer treatment

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Introduction

Another complication that further exacerbates the successful cancer treatment is cancer recurrence during the post-treatment regime. Breast cancer, lung cancer, and ovarian cancer are a few of the most recurring cancer types (1–9). These cancers may recur in the same or nearby tissues, or metastasize to distant tissues and organs. Even after successful treatment of the tumor cells, very few cancer cells remain unscathed as these cells are small in number and size, do not proliferate, and remain undetected in follow-up clinical investigations (10). These dormant cells, viable yet non-proliferating, can remain undetectable for several years before recurring in patients who have received cancer therapies. They can also undergo cell cycle dormancy and have enhanced DNA repair ability (11). A study by Albrengues J. *et al*. have shown that NETs are involved in the reawakening of dormant cancer cells in mice (10).

Globally, researchers are working rigorously to find new and safe compounds for the treatment of cancer with minimal harmful effects. Moreover, it is important to look for novel strategies to prevent cancer recurrence as well. In this regard, APC and LF could be potential candidates that could be useful in treating cancer and prevent its recurrence. This review summarizes the anticancer activity of LF and APC. The role of NETs and derived proteins such as neutrophil elastase, Cathepsin G, calprotectin, and matrix metalloproteinase 9 (MMP9) in the progression and metastasis is discussed. Besides, the role of NETs in the reawakening of dormant cancers has been elucidated. Finally, how LF and APC help in the inhibition of proliferation, migration, metastasis, and recurrence of cancer are discussed. Furthermore, we have described the limitations and emphasized understanding the mechanism as well as limitations, which would facilitate developing new therapies for cancer and its subsequent control.

NETs in cancer progression and reawakening

Neutrophils are innate immune cells that kill invading pathogens by phagocytosis, releasing cytotoxic enzymes and proteases or forming NETs (12). NETs are highly decondensed chromatin decorated with cytotoxic enzymes and proteases like neutrophil elastase, calprotectin, MMP9, cathepsin G and LF released into extracellular space to kill microbial pathogens (10,12,13). The formation of NETs entails three major processes. At first, histone protein H3 undergoes citrullination by the enzyme protein arginine deiminase 4 (10,12,14). Citrullination of histone causes the unfolding of chromatin (15). Then 30 different neutrophil proteins attach to decondensed chromatin (10,12). This is further followed by the release of the associated chromatin-enzyme complex into extracellular space after rupturing the plasma membrane (10). Microbial infection, inflammation, and instillation of lipopolysaccharide have shown the formation of NETs. NETs are associated with the cause of different diseases in humans. Studies have shown the role of NETs in COVID-19, atherosclerosis, thrombosis in lungs and kidneys of sepsis patients, delayed wound healing in diabetic patients, rheumatoid arthritis, periodontitis, and systemic lupus erythematosus (Figure 1) (10,12,16,17).

Reports also depict that apart from being associated with the diseases mentioned above, NETs are also associated with cancer progression, development and recurrence (18,19). The formation of NETs due to post-surgery stress advances the progression of cancer (20). In cases of post-surgery systemic infections, NETs have been shown to aid in the advancement of tumors (21). Cools-Lartigue et al., have concluded that NETs and different proteins associated with NETs play a critical role in the adhesion, proliferation, invasion, migration and angiogenesis of tumor cells (22). It has been postulated that NETs create a microenvironment

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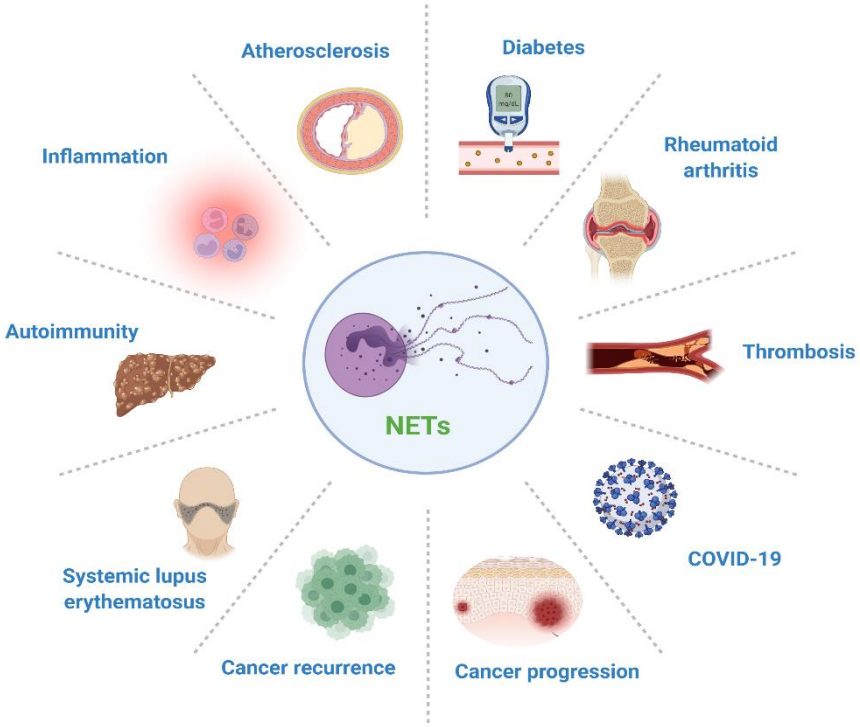

Fig.1. Association of NETs with various diseases. Figure Created with BioRender.com.

for the progression of cancer cells by sequestering tumor cells and bringing them close to different neutrophil-derived proteins like neutrophil elastase, cathepsin G, and MMP9 (22). Similarly, separate studies on the role of different neutrophil-derived proteins rather than the whole NETs have shown the critical role of these proteins in different steps of the metastatic cascade. MMP9, a zinc-dependent endopeptidase, plays a vital role in tumor growth by augmenting proliferation rate, angiogenesis process, invasion and migration of tumor, and lessening tumor cell apoptosis (22–24). The enzymatic activity of Cathepsin G, another neutrophil-derived serine protease, has been shown to be associated with tumor angiogenesis and helps in the aggregation of tumor cells to form metastatic foci at secondary sites mediated by the insulin-like growth factor 1 (IGF1) signaling (22,25,26). Neutrophil elastase (NE) also augments the migratory activity, progression and proliferation of tumor cells by the release of growth factors like transforming growth factor α (TGFα), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (27,28). NE exhibits tumorigenic activity by the activation of epidermal growth factor receptor (EGFR) and toll-like receptor (TLR); activation of phosphoinositide 3 kinase (PI3K)-Akt pathway and inactivation of tumor suppressors like EMILIN 1 (Elastin Microfibril Interfacer 1) and TSP-1 (Thrombospondin 1), as well as promote angiogenesis by stimulating the VEGF release from the tumor cells (29). Furthermore, Calprotectin, a calcium-binding protein of S-100 protein family, generally present in NETs at low extracellular levels, has been found to assist in angiogenesis and migration of tumors by activating NF-kB (30,31). Activated NF-kB further upregulates the expression of different genes playing an essential role in the angiogenesis and

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migration of tumors (31). Thus, based on the studies mentioned above, it can be concluded that NETs and NETs-derived proteins can favor the proliferation, progression and metastasis of tumors.

Not only NETs and their associated proteins help in tumor progression, but they also help in the reawakening of dormant cancer cells. Dormant cancer cells are cancer cells that spread from the primary tumors to local or distant tissues, remain clinically undetectable for years and eventually recur after a prolonged time (10). Cancer recurrence is quite common in many cancer types such as breast cancer, prostate cancer, lung cancer, cervical cancer and ovarian cancer patients (7–10,32). The study by Albrengues et al. has shown that the formation of NETs upon exposure of tobacco smoke or nasal instillation of lipopolysaccharide in breast and prostate cancer mice models engendered the proliferation of dormant cancer cells (10). The awakening of the dormant cancer cells occurs via the cleavage of laminin found in the extracellular matrix (ECM) by neutrophil elastase and MMP9. Cleavage of laminin by these proteins found in NETs further activates the integrin-mediated signaling pathway and incites the proliferation of dormant cancer cells (10). Thus, the role of NETs in cancer recurrences even after successful treatment has been corroborated.

APC and its anticancer activity

Cancer is associated with the prothrombotic state (abnormalities in the coagulation system due to acquired or inherited molecular defects that espouse the formation of thrombus in blood vessels), which helps in the progression and metastasis of tumors (33,34). A prothrombotic state occurs in cancer patients due to the release of procoagulant factors, impaired fibrinolysis, reduction in coagulation inhibitor production, and production of inflammatory cytokines (34). Based on the review of different clinical trials, Spek and Arruda showed that abnormal blood clots aid in the progression of cancer by increasing metastasis and helping in the adherence of cancer cells to endothelial cells and platelets (35). As it has been established, the prothrombotic state helps in cancer progression; anticoagulant therapy can be a potential way to thwart the hypercoagulable state-mediated metastasis in cancer patients (34–36). In this regard, a natural anticoagulant APC can be a promising option. APC is a serine protease anticoagulant that inactivates coagulation co-factors Va and VIIIa to inhibit the further generation of thrombin necessary for an effective clotting process (35,36). Along with the anticoagulant activity, this 56 kDa, vitamin K dependent serine protease also possesses anti-inflammatory and cytoprotective properties (37). Furthermore, APC has a beneficial impact on the heart during ischemia and reperfusion. Additionally, APC prevents ischemia and reperfusion induced cardiac Endothelial protein C receptor shedding, which is important for preventing cardiac damage in aging (38). A recent study has reported that APC improves idiopathic membranous nephropathy by affecting the apoptosis of podocytes (39).

In the study by Wilts IT et al., it was concluded that the decreased activity of APC is related to increased deaths in cancer patients at advanced stages (40). Similarly, a low level of APC is associated with higher mortality in patients with advanced stages of pancreatic, lung and prostate cancer (41). Another study by Roselli M et al. showed that the activity of APC was challenged in about 20% of patients who have blood cancer (BC) (42). Women with the advanced stage of BC or distant metastases had more impaired APC function than women with stage I and stage II BC (42). They also concluded that due to damage to the activity of APC, there is an abnormal clot formation in the blood vessels of cancer patients, which will further

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exacerbate the disease by promoting tumor growth and metastasis (42). The administration of recombinant human APC in experimental lung metastasis mice models has been shown to reduce the metastasis process by inhibiting the adhesion and migration of tumor cells (Figure 2) (43). Studies have reported that the APC and APC-2Cys (a mutant of APC with low anticoagulant activity) considerably reduced the migration and metastasis of the B16F10 murine melanoma cells by inhibiting transendothelial migration of malignant cells in mice (44,45). APC has also hindered the extravasation of cancer cells by enhancing the vascular endothelial barrier through cross-activation and sphingosine-1-phosphate-receptor-1 (35,46). The interaction of APC with endothelial protein C receptor on cancer cells, likely reduces the endothelial adhesion molecules expression, like P-selectin which is important for tumor cell-endothelium interactions during extravasation, ultimately hindering the cancer cell dissemination (44). Similarly, the monoclonal antibodies against APC have increased the extravasation of cancer cells in the murine model (35). These studies show the decreased level of APC and impaired function of APC are associated with cancer progression and cancer-related mortality. Thus, these studies imply that the APC plays an important role in preventing prothrombotic state-mediated cancer progression and deaths; and that APC-based anticoagulant therapy can be an effective approach to mitigating the damage caused by cancer. Although this approach seems plausible, the limitations faced in using APC therapy cannot be ignored. Though initially, the use of recombinant APC (rAPC) showed a reduction in mortality among sepsis patients, the therapeutic use of rAPC in sepsis patients was discontinued in 2011 as it did not show any benefit after 10 years of clinical trials and it also increased risk of bleeding in patients (37,47). The plethora of opportunities provided by genetic engineering can be seminal in designing rAPC with low anticoagulant activity, to mitigate the risk of bleeding, and better anticancer activity. By using a similar approach, Wang Y et al. used recombinant 3K3A-APC to study its neuroprotective activity (48). 3K3A-APC is a genetically engineered analog of APC with low anticoagulant activity but maintained cytoprotective, cell signaling, and anti-inflammatory (48). In their study, Wang Y et al. showed the neuroprotective activity of 3K3A-APC (48). Thus, a similar approach can be used to engineer variant APC with a low risk of bleeding and high antitumor property.

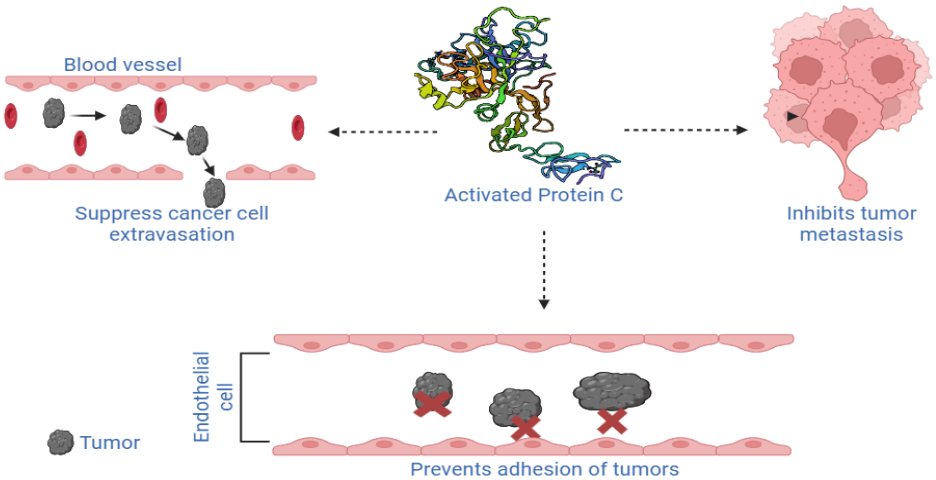

Fig.2. Anticancer activities of APC. Figure Created with BioRender.com.

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APC against inflammation and NETs mediated cancer progression and recurrence

It has been established in different studies that inflammation induces the formation of NETs and NETs are associated with cancer progression and reawakening of dormant tumors (10,20,22). So, the role of APC, which has already shown the potential to prevent prothrombotic state-mediated cancer progression, in preventing NETs-mediated cancer progression can be crucial. As inflammation induces NETosis the activity of APC against inflammation seems imperative. Sarangi PP et al. showed in their study that the recombinant APC could mitigate inflammation in septic mice models by binding to integrin, VLA-3, present on the surface of neutrophils (49). Neutrophils need integrins for migration, adhesion and signaling (49). Nazir et al. also showed the anti-inflammatory activity of APC, where it restricted the inflammasome signaling necessary for detecting pathogens (50). A recombinant APC treatment inhibited inflammasome activation and showed anti-inflammatory activity in mice models of uveitis (51). In the study by Healy et al. the proteolytic activity of APC inhibited the NET formation and the activation of neutrophils in baboons used as bacterial sepsis models (37). The APC-mediated inhibition of NETosis involves the binding of APC to neutrophil receptors like β-integrin, Macrophage-1 antigen (Mac-1), Protease-activated receptor 3 (PAR3) and endothelial protein C receptor (EPCR), which in turn activate cytoprotective signaling (37). Based on these different studies, where APC has shown anti-inflammatory activity and properties to inhibit NET formation in vitro and in vivo, the potential role of APC in curbing NET-associated cancer progression and recurrence is substantiated. However, so far no study has reported the direct potential of APC in preventing NETs-mediated cancer progression and recurrence. Hence, further studies relating to the role of APC against NET and NET-derived proteins in vitro, and cancer models will be beneficial to corroborate the anticancer activity of APC. Regarding the activity of APC against neutrophil-derived proteins, in a study by Tanaka et al. APC mitigated the lung damage caused by neutrophil/leukocyte elastase treatment in mice (52).

LF and its anticancer activity

LF is a glycoprotein found in breast milk, tear, sweat, the bile of mammals and humans saliva and plasma (53, 54). This 80 kDa iron transport protein with 703 amino acids plays an essential role in host defense because of its antibacterial, fungicidal, antiviral, antioxidant and immunomodulatory activity (54–56). Various studies conducted in recent years have shown the anticancer activity of LF (Figure 3). These studies on animal models and cell lines of different cancer types have demonstrated the ability of LF to inhibit the proliferation, differentiation, migration and angiogenesis of tumor cells (54, 57, 58).

There are different mechanisms exerted by LF to inhibit the progression of cancer. One of the mechanisms is to help control the generation of reactive oxygen species (ROS). LF has two domains with iron-binding sites. Thus, by binding to iron, which generates ROS by Fenton and Haber-Weiss reaction, LF limits ROS generation and prevents possible damage to different macromolecules like protein, RNA, DNA and lipids (54). ROS causes oxidative stress which in turn is related to cancer cell proliferation, invasion, survival, angiogenesis and metastasis (59).

Another mechanism is to trigger the apoptosis of cancer cells. A study by Luzi C et al., showed LF caused the reduction of glutathione (GSH) levels in HeLa cell lines (54). The reduction of GSH caused the activation of Caspase-3 and Caspase-9 (54, 57). Caspase-3 and Caspase-9 play an important role in mediating apoptosis (54, 57). Similarly, LF has inhibited the expression of survivin protein and induced apoptosis as

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well as reduced tumor migration and invasion without any cytotoxic effects on normal cells (60). Survivin is an inhibitor of apoptosis and is highly expressed on the plasma membrane of very metastatic cells (60). Thus, inhibition of survival by LF plays an important role in preventing cancer proliferation and progression.

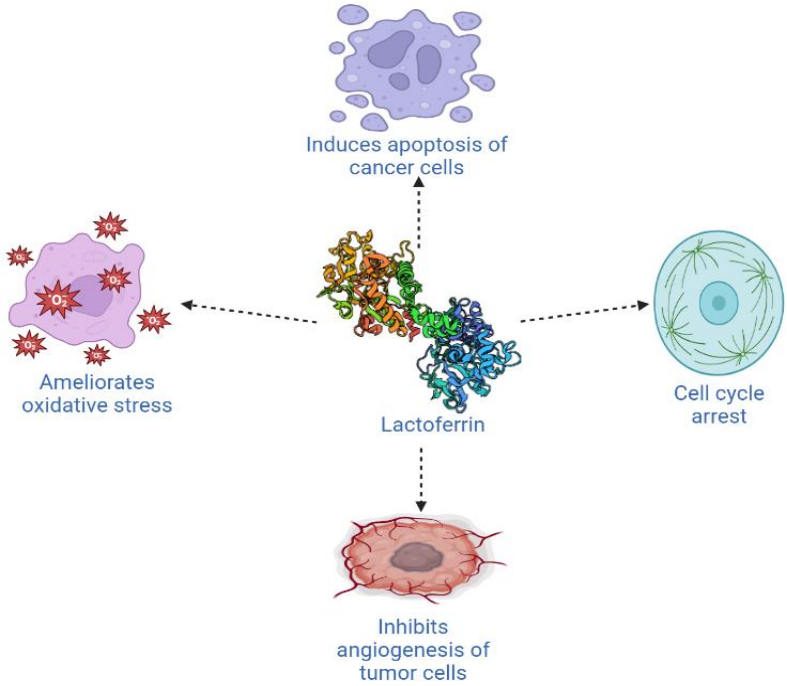

Fig.3. Different roles of LF related to cancer. Figure Created with BioRender.com.

Moreover, LF hinders cancer cell proliferation by arresting the cell cycle. LF activates different cell signaling pathways which further upregulates P53, p21, ATM, CHK and BRC1 signaling pathways (57). The upregulation of these pathways is associated with cell cycle arrest (57). LF has also shown the ability to prevent the growth of new blood vessels necessary for the growth of tumors. In the study by Li HY et al., LF downregulated the expressions of VEGFR 2 and VEGFA (55). VEGFR and VEGFA are essential for the angiogenesis of tumors. Further, LF was able to bind different subunits of VEGFR 2 and VEGFA, thus preventing the formation of their functional multimers and downregulating their expression (55). LF also inhibited the VATPase, imperative for maintaining the acidic environment around tumors and helping in the migration and metastasis of tumors (58). V-ATPase is a proton pump present on the plasma membrane of highly metastatic cells, whereas, in normal cells, it is mainly displayed in intracellular compartments (58). LF's anticancer properties, such as cell cycle regulation, promotion of apoptosis, and inhibition of migration, require direct recognition and differentiation between cancerous and normal cells. This recognition may involve a primary interaction with specific cancer cell surface receptors or a secondary interaction with the regulation of differential intracellular networks (61). LF's anticancer action may be attributed to cell signaling and recognition through the glycans within its structure. Conversely, cancer cells typically contain high levels of sialic acid, glycosaminoglycan and proteoglycan, which are known to interact with LF. This interaction may activate additional signaling pathways that lead to detrimental effects on the cells (62).

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LF has also shown synergistic effects in combination with other anticancer drugs. In breast cancer-bearing mice, the nanoconjugate formed by conjugation of LF with docetaxel and celastrol exhibited superior in vivo anti-tumor effectiveness in a synergistic manner, which was evident by a decrease in tumor volume, improved survival rate, and notable suppression of Ki-67, TNF-α, COX-2, and NF-κB p65 expression levels in comparison to the group that received free docetaxel and celastrol, a combination of docetaxel and celastrol and positive control (63). Similarly, another study has also reported the synergistic effect of LF in improving the effectiveness and safety of docetaxel in prostate cancer Mat Ly Lu cells treatment (64). In another study, the administration of LF and epirubicin combination to solid Ehrlich carcinoma-bearing mice lowered tumor volume, and increased survival rate as well as tumor inhibition rate in comparison to the epirubicin mono treated group and tumor control group (65). LF enhanced the lung cancer cell growth inhibition of etoposide where their combined treatment caused apoptosis induction, cell cycle arrest and achieved the same anticancer effect with a 10-fold reduced dose of etoposide. Additionally, the combination treatment reduced the cytotoxic effect of etoposide on endothelial cells (66).

Additionally, nanoformulations of LF have also shown anticancer properties. A study has reported the superior anticancer activity of a nano combination formed by embedding LF in iron nanoparticles against different cancer cell lines (67). The LF nanoliposome showed more significant activity in inhibiting the Caco-2 cells than the LF alone (68). The LF- gallium (Ga, III) isopropyl-2-pyridyl-ketone thiosemicarbazone compound (C4) nanoparticle had a higher capability to hinder tumor growth compared to LF or C4 used alone by activation of the immune system, inhibition of tumor angiogenesis and killing of cancer cells (69). The graphene oxide-LF nano combination showed anticancer properties against TC-1 lung cancer cells by inducing apoptosis and cell cycle arrest. Furthermore, the nano combination significantly inhibited the growth of tumors in mouse models of lung cancer (70). LF-doxorubicin-mesoporous maghemite nanoparticles inhibited the proliferation of breast cancer cells and showed anti-metastatic properties against breast tumors in animal models (71).

LF against inflammation and NETs mediated cancer progression and recurrence

Inflammation plays an important role in cancer initiation, progression and NETs formation (20, 22, 72). In the study by Sugihara Y et al., liposomal bovine LF inhibited the expression of tumor necrosis factor-α and cell growth (72). They concluded that the finding strengthens the hypothesis of LF preventing carcinogenesis by affecting inflammation (72). LF inhibited the formation of NETs in vitro and in vivo (13). Both endogenous (LF as a component of NETs) and exogenous (intravenous administration) LF suppressed the release and formation of NETs (13). Though endogenous LF can inhibit NETs formation; it lacks the ability to inhibit excess NETs formed during disease conditions (13). The administration of exogenous LF can be a good prospect to prevent NETosis in pathological conditions. The sequence of 25 positively charged amino acids and charge-charge interaction between LF and NETs are important for preventing the formation of NETs (13). Similarly, LF administration reduced the level of MMP9, a component of NETs, in women undergoing amniocentesis (73). Based on these studies, it can be hypothesized that LF can be a promising agent to prevent the NETs-mediated cancer progression and reawakening. However, so far no study has shown the direct potential of LF in inflammation and NETs mediated cancer progression and recurrence. Further studies are needed to corroborate the hypothesis.

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Limitations in the therapeutic use of APC and LF and future directives

As LF and APC are natural products, they are theoretically supposed to have low side effects and chemoresistance (58). Nevertheless, there are possibilities that they may have side effects when used for cancer therapy. Hence, future studies need to determine their safety and toxicity for human use. Furthermore, they can be used in combination with approved anticancer drugs for improving their effectiveness. The possibility of using them as therapeutic drugs has other limitations as well. The action of LF is dependent on cell location. Based on its location, LF can have a cytotoxic effect or help in the proliferation of tumors. In the study by Tammam SN et al., LF showed cytotoxicity against glioma in cytoplasmic delivery but caused the proliferation of cancer cells when LF was targeted to the nucleus (74). There is an increased risk of infection when the formation of NETs is inhibited. In this regard, LF itself possesses antibacterial, fungicidal and antiviral activity; and APC has increased autophagy in sepsis and improved the antibacterial activity of levofloxacin (54, 55, 75, 76). There has been a report of LF instability upon administration in clinical studies (13).

APC increases the risk of bleeding in patients with severe sepsis (37, 47). The plethora of opportunities provided by genetic engineering can be seminal in designing rAPC with low anticoagulant activity to mitigate the risk of bleeding and better anticancer activity. Further studies should be directed towards understanding the role and mechanisms of LF and APC against NETs derived proteins like calprotectin, neutrophil elastase, and cathepsin G. These studies will help us decide on possible targets of APC and LF in inhibiting the NETs, and further NETs mediated cancer progression and metastasis. Further, studies are also needed to design strategies to improve the stability, and anticancer activity without jeopardizing the immune activity of neutrophils during the administration of LF and APC. LF has been used as the carrier and targeting ligand molecule, and APC can cross blood-brain-barrier and neuroprotective properties (77–79). Furthermore, in the future, the anticancer property and the ability to suppress the NETs-mediated cancer progression of different peptides obtained from LF against various cancers can be explored (80). Based on these assumptions, future development of LF-APC conjugates with their anticancer, neuroprotective and blood-brain-barrier crossing properties can be hypothesized for the treatment of glioma and other brain cancer. Moreover, as so far no study has reported the direct potential of LF and APC in preventing NETs mediated cancer progression and recurrence future studies should also focus on determining these potential of LF and APC.

Conclusion

The rate of new cancer incidences and mortality is increasing rapidly worldwide. Further, the global cancer burden is exacerbated by the recurrence of cancer in patients after successful clinical treatments. Most of the current therapies have harmful side effects, and some tumors have developed resistance to chemotherapy and radiation therapy. So, it becomes imperative to find novel therapies for cancer without harmful effects. In this regard, LF and APC have shown potential. Prothrombotic state, inflammation and NETs are associated with the progression and recurrence of cancer. Activated protein C with its anticoagulant activity can overcome the cancer progression mediated by the prothrombotic state. APC has inhibited inflammation, and mitigated damage caused by NETs derived proteins like neutrophil elastase and NETs formation in vitro and in vivo. Similar properties have also been shown in LF. LF has inhibited the

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proliferation, differentiation, migration, and angiogenesis of tumor cells. Further, LF has the ability to inhibit ROS generation and induce apoptosis and cell cycle arrest in cancer cells. The charge-charge interactions between LF and NETs have been able to prevent NETs formation in vitro and in vivo.

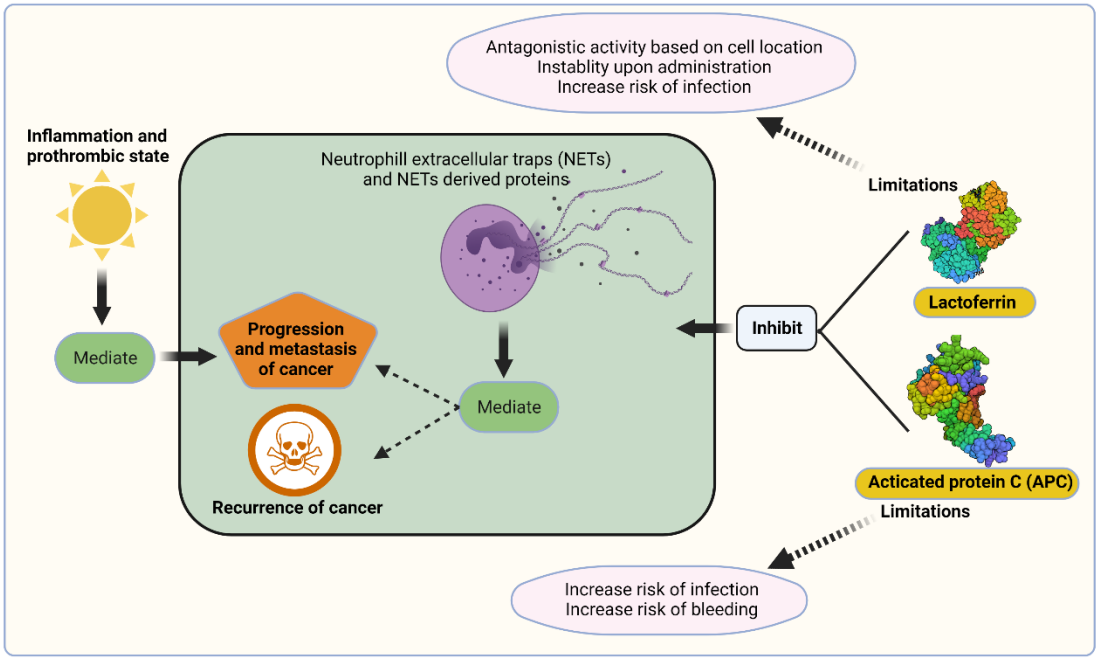

Fig.4. LF and APC prevent the recurrence of cancer. LF a glycoprotein and natural anticoagulant APC inhibits the NETs and its associated proteins. NETs are associated with the progression, development and recurrence of cancer. Cancer is also mediated by the inflammatory response. Further, the limitations of the LF and APC have also been highlighted (arrowhead). Figure Created with BioRender.com.

Though the studies discussed in the review show the great potential of LF and APC as effective anticancer substances, their previous therapeutic use has shown some limitations like high risk of bleeding, infection, instability and antagonistic activity at different target sites as shown in (Figure 4). Future studies need to be targeted toward addressing these limitations to make the use of LF and APC more effective. Genetic engineering to develop recombinant APC and LF with better activity and low side effects is a field that needs further exploration. Further studies should be directed towards understanding the role and mechanisms of LF and APC against NETs derived proteins like calprotectin, neutrophil elastase, and cathepsin G. These studies will help us decide on possible targets of APC and LF in inhibiting the NETs, and further NETs mediated cancer progression and metastasis. The hypotheses of LF-APC conjugate need to be tested against glioma and other cancer *in vitro* and *in vivo* models.

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